MOLECULAR GENETIC INVESTIGATIONS OF HARBOR SEAL STOCK STRUCTURE IN ALASKA

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INTRODUCTION

Over the past two decades, harbor seals (*Phoca vitulina richardsi)* have declined dramatically in some areas in Alaska (Pitcher 1990, Lewis *et al*. 1996, Frost *et al*. 1999, Jemison and Pendleton 2001, Small *et al*. 2001). In contrast, seal numbers in other regions of the State have remained stable or increased during this same period (Lewis *et al*. 1996, Small *et al*. 2001) The declines prompted the Marine Mammal Commission to list Alaskan harbor seals as a species of special concern. Differences in trends led the National Marine Fisheries Service (NMFS) to create three large provisional management stocks (Hill *et al*. 1997), but a need for better defined management units persists. An understanding of the scale at which population subdivision may act and the amount of dispersal (exchange) between areas is critical to the definition of biologically meaningful management units. Recent telemetry studies have revealed much about movement patterns in Alaskan harbor seals (Pitcher and McAllister 1981, Frost *et al*. 1997, 1998, 1999, Swain *et al*. 1996, Swain and Small 1997, Small and Ver Hoef 2001, Rehberg and Small 2001) but can tell us, as yet, little about the rate and mode of dispersal. Genetic analyses offer the most viable approach to estimating levels of dispersal and thus defining management units in this species. Identifying stock boundaries in this way will help in estimating population size, assessing levels of take, and interpreting trend counts. Examination of patterns of variation in markers with different modes of inheritance (maternal vs. bi-parental) can provide insights into differences in breeding and movement behaviour among areas and elucidate the relationship between gene flow and dispersal. Whereas significant levels of genetic differentiation indicate that dispersal is limited, typically on the order of a few individuals per generation, estimates of differentiation at genetic markers that are considered to reflect contemporary patterns of dispersal could be used with estimates of effective population size, N_e, to estimate actual dispersal rates.

Scientists at the Southwest Fisheries Science Center have been using molecular genetic techniques to investigate population subdivision and movement patterns of harbor seals in Alaska. Variation in both mitochondrial and nuclear markers is being examined to resolve population structure and estimate levels of dispersal that will provide the framework for delineating stock boundaries. The different modes of inheritance of the two types of marker may also determine whether separate stocks are demographically and/or reproductively independent by distinguishing between actual (*i.e*., emigration) and effective (*i.e*., interbreeding) dispersal. An extensive body of data has been collected for both marker types and initial analyses have revealed that Alaskan harbor seals do not conform to a single

panmictic population. The fact that harbor seals are distributed almost continuously throughout their Alaskan range, however, has made it difficult to discern at what scale population subdivision is acting and where sub-population boundaries lie. This has necessitated the development of new techniques for analyzing the genetic data in order to resolve population structure and identify management units.

This report summarizes findings on a number of projects that were funded, in part or in total, from the National Oceanic and Atmospheric Administration (NOAA) grant to the Alaska Department of Fish and Game (ADF&G). Efforts during the previous 9 months focused on: (1) continuing the collection of mtDNA data, (2) developing two methods for analyzing population genetic structure in a continuously distributed species, (3) increasing the number of loci screened and expanding the coverage of the microsatellite analysis, (4) developing lab techniques to extract DNA from alternative sample types in order to increase size of coverage, and (5) estimating effective population size, N_e, through a molecular genetic investigation of mating systems in North Pacific harbor seals. Much of this work is at a critical stage of data analysis and interpretation and so it was decided to prepare this report as a series of summaries of the first four projects. Details of laboratory and analytical methods, as well as final results and interpretation, will be presented in a number of forthcoming manuscripts and theses, and subsequent reports.

Mitochondrial DNA analysis

The analysis of sequence variation within the mtDNA control region continued with the addition of samples from a number of key areas, including Southeast Alaska, Prince William Sound, the Kenai Peninsula, the Kodiak Archipelago, and the Pribilof Islands. The total number of samples sequenced to date, including a small number of outlying samples from Japan, Russia and California, is 749. The methods used have been described in previous reports (Westlake and O'Corry-Crowe 1997, O'Corry-Crowe *et al*. 1999). Haplotype variation is high in Alaskan harbor seals and the pattern of mtDNA differentiation is generally clinal in nature with the greatest differences found among the extreme ends of the range (Westlake 1997). When the range of this species is divided into the three provisional PBR (Potential Biological Removal) areas or stocks suggested by the Alaska Scientific Review Group (SRG) and adopted by NMFS (Hill *et al*. 1997), highly significant genetic differences were found between all three (Table 1). However, although these findings confirm the presence of population subdivision in Alaskan harbor seals, it should be stressed that they may not reflect the true spatial scale at which this structure is acting and that the current PBR boundaries may not adhere to natural barriers to movement (see below).

Analysis of population structure in a continuously distributed species

Dealing with a continuously distributed species, such as harbor seals in Alaska, creates a number of problems for the would-be investigator of population genetic structure. The first is where to begin looking for potential subdivision. The scale at which population structure acts, let alone the location of sub-population boundaries, is often difficult to discern in a continuously distributed species. A second problem is that within a continuously distributed species, structure is often clinal with the greatest genetic differences occurring among sub-populations at the extremes of the range such that the greatest genetic differentiation is found when the range is divided equally into a few large areas, even when population structure actually occurs on a smaller spatial scale (Taylor and Dizon 1999). This is because the neighboring areas contain the most geographically and genetically distinct as well as most similar individuals. Furthermore, the power to detect differentiation is increased by the relatively large sample

sizes within the small number of areas being compared. Thus, there is a danger to starting too large with your initial hypotheses and therefore missing the underlying structure that may exist on a smaller geographic scale.

Table 1. Genetic differentiation among the three provisional PBR areas. Both genetic distance-based (Φ_{st}) and haplotype frequency-based (F_{st} and χ^2) statistics were used. Significance values (P) are based on 1000 randomizations of the data set.

PBR area		Bering Sea	Gulf of Alaska	Southeast
	n	49	434	239
	p-value			
Gulf of Alaska	Φ_{st}	0.016		
	F_{st}	0.000		
	χ^2	0.000		
Southeast	Φ_{st}	0.000	0.000	
	F_{st}	0.000	0.003	
	χ^2	0.000	0.000	

Given the problems of resolving population structure in a continuously distributed species, analysis of the genetic data has proved challenging. Considering the distribution pattern of harbor seals in Alaska and the nature of mtDNA variation across their range, it is not surprising that significant genetic differentiation was found among the initial PBR boundaries. We are taking two quite distinct, but complementary, approaches to analyzing the mtDNA data and identifying management stocks. Improvements to both methods are currently being finalized and a comparison of findings from both approaches will be done presently. Here we have chosen to give an outline of each approach and to delay presentation of findings until the analyses are completed.

Approach I

In this approach, the problem of determining at what geographic scale sub-structure may be working is dealt with by reviewing all the information available on harbor seal movement patterns and integrating it with data on other factors that may influence or reflect population sub-division (*i.e.*, abundance, distribution, and differences in pupping season and morphology) to come up with a series of starting strata to be tested for genetic differentiation. The problem of potentially missing structure by starting at too large a geographic scale is overcome by purposely defining these initial strata on a spatial scale that is considered conservative. Once the first round of analysis is complete a decision is made regarding which areas can be combined and the analysis of genetic subdivision is run again on the modified set of strata. Successive rounds of analyses followed by lumping of neighboring strata ultimately results in a number of well-supported strata representing demographically discrete sub-populations among which dispersal is limited.

The statistical power of the data to detect underlying subdivision, however, is often a concern when deciding which areas to combine. Given a perfectly sampled study, absence of genetic subdivision

alone may justifiably be considered grounds for combining two neighboring areas in a subsequent round of the analysis. However, if sample size is low in a particular area, the lack of significant differentiation may be the result of poor sample size. Thus, it is necessary to be able to distinguish between the absence of genetic differentiation due to inadequate sampling versus truly no underlying population structure when deciding whether or not to combine strata. A further concern may be whether the particular marker under examination possesses the appropriate level of variation to detect subdivision at the level being studied.

We are currently investigating ways of assessing the weight of these and other factors that may need to be considered in the decision process, including re-evaluation of movement data and likely historical relationships among areas that may still have an influence on the genetic picture seen today.

Approach II

An alternative approach to the identification of management stocks from the mtDNA data is that of Martien and Taylor. This approach allows the genetic data itself to find the boundaries by searching for significant discontinuities in patterns of genetic variation on a geographic scale. Neighboring strata are clustered in successive rounds of the analysis if no differentiation is found. This *a posteriori* clustering of strata based on genetic differentiation alone contrasts with the traditional hypothesis-based methods of Approach I where strata are established *a priori* and where decisions to lump are not based solely on the absence of detectable differentiation. This second approach may do better at resolving the evolutionary relationships among areas and thus detecting population structure not immediately apparent from the movement data, etc. Analyses are at an advanced stage and will be compared with the findings from the *a priori* approach as soon as that analysis is completed. Details of this approach are currently being written up for publication.

Microsatellite Analysis

As well as examining variation within mtDNA, we are examining patterns of variation within highly variable nuclear markers, called microsatellites, to determine stock structure in Alaskan harbor seals. Research from January 1997 to June 1999 was concerned with developing lab protocols to: (1) screen for variation at several microsatellite loci, (2) compare levels of variation found at these loci with levels found at microsatellite loci in other harbor seal populations, and (3) determine the utility of these hypervariable markers in identifying management stocks of harbor seals by investigating movement patterns, breeding behaviour and gene flow within and between areas. PCR conditions were optimized for a total of 8 independent loci (O'Corry-Crowe 1997). The level of polymorphism varied greatly among loci, but was found to be generally higher than levels in some European harbor seal populations (O'Corry-Crowe 1998). Seven of the loci have proven informative for population subdivision studies in Alaskan harbor seals with strong differentiation signal occurring across large geographic areas (O'Corry-Crowe *et al*. 1999).

Analysis of samples at these 7 loci has continued with the addition of samples from the Pribilof Islands, Yakutat and Icy Bays as well as Southeast Alaska, Prince William Sound, and the Kodiak Archipelago. Furthermore, PCR conditions were optimized for 4 new microsatellite loci (Table 2). Two were originally typed on grey seals, *Halichoerus grypus*, (Hg4.2 and Hg6.3, Allen *et al*. 1995), one on North Atlantic harbor seals, *P. v. vitulina*, (SGPV11, Goodman 1998) and one on elephant seals, *Mirounga* sp., (BG) which was previously found to work on North Atlantic harbor seals (Gemmell *et al*. 1997). To date over 250 samples from the entire Alaskan range of the species have been analyzed for

variation at between 7 and 11 independent loci. As with mtDNA, initial analysis has revealed structure on a broad geographic scale. Seals from Bristol Bay, for example, are genetically distinct from seals in the Gulf of Alaska. We have found that the number of samples greatly influences the reliability of estimates of genetic subdivision. Small sample size increases the variance in the test statistic thus increasing the probability of a type II error of falsely not rejecting the null hypothesis of panmixia. There is therefore a need to increase sample size from a number of areas.

Table 2. The four microsatellite loci optimized for Alaskan harbor seals in this study, the species upon which they were originally typed, their repeat motifs, the number of alleles detected to date, and the original publications.

Locus	Species	Repeat motif	No. of alleles	Publication
Hg 4.2	*H. grypus*	CA	6	Allen *et al*. 1995
Hg6.3	*H. grypus*	CA	6	Allen *et al*. 1995
SGPv11	*P. vitulina*	CA	9	Goodman 1998
BG	*Mirounga* sp.	GGAAA	9	Gemmell *et al*. 1997

Alternative approaches to sample acquisition

Harbor seal samples for genetic analysis have, up to now, consisted almost entirely of tissues (skin, muscle, liver) taken directly from live or dead animals. These samples come primarily from subsistence harvest and tagging operations, and as such, sample coverage is dependent on where these activities take place. Because of their typically skittish nature and their tendency to haul out on relatively inaccessible coastlines, harbor seals are often difficult to catch and sample. This difficulty in directly sampling harbor seals explains, in part, the gaps in sampling along their Alaskan range and prompted us to investigate a number of indirect methods to sample acquisition.

Beginning in 1997, a project was initiated to develop laboratory methods to extract DNA of sufficient quantity and quality for PCR and sequence analysis from a number of alternative sources of genetic material, namely: scat, shed hair, formalin-fixed tissues, birth evidence, and shed blood. Since then, a protocol has been developed to extract DNA from birth evidence and from blood (that is shed either during birth or as a result of male-male fighting) of a quality adequate enough for amplification and sequencing of the mtDNA control region and analysis of variation at several microsatellite loci. Secondly, we have been successful in extracting, amplifying and sequencing mtDNA from hair shed by seals while hauled out on glacial ice in Glacier Bay. DNA has also been extracted successfully from scat, although the universal utility of our mtDNA primers to many vertebrate taxa have limited their use in amplifying and sequencing the host DNA as they also amplify the DNA of prey species present in the scat. Finally, the fixing of tissues in formalin has long been accepted as excluding such samples from genetic analysis, primarily because of the difficulty in extracting DNA of high

enough quality in sufficient quantity. Problems may also arise at the amplification (PCR) and sequencing stages. Considering the wealth of marine mammal samples that have been collected over several decades and fixed in formalin and subsequently preserved in alcohol, SWFSC initiated a project to develop methods to extract DNA from formalin-fixed tissues. Initial results are promising. We successfully extracted and sequenced mtDNA from 9 harbor seal samples collected from the Pribilof Islands more than 20 years ago, more than doubling our sample from this area.

These projects have expanded greatly our ability to collect samples for genetic analysis and fill in gaps in our sampling coverage of Alaskan harbor seals. Such non-invasive approaches also avoid the possible disruptive effects of traditional sampling methods on harbor seal behaviour. Further optimization of protocols is required, however. In some tissue types, sequencing is possible but microsatellite amplification is proving problematical.

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